Transporters of arsenite in rice and their role in arsenic accumulation in rice grain

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Arsenic poisoning affects millions of people worldwide. Human arsenic intake from rice consumption can be substantial because rice is particularly efficient in assimilating arsenic from paddy soils, although the mechanism has not been elucidated. Here we report that two different types of transporters mediate transport of arsenite, the predominant form of arsenic in paddy soil, from the external medium to the xylem. Transporters belonging to the NIP subfamily of aquaporins in rice are permeable to arsenite but not to arsenate. Mutation in OsNIP2;1 (Lsi1, a silicon influx transporter) significantly decreases arsenite uptake. Furthermore, in the rice mutants defective in the silicon efflux transporter Lsi2, arsenite transport to the xylem and accumulation in shoots and grain decreased greatly. Mutation in Lsi2 had a much greater impact on arsenic accumulation in shoots and grain in field-grown rice than Lsi1. Arsenite transport in rice roots therefore shares the same highly efficient pathway as silicon, which explains why rice is efficient in arsenic accumulation. Our results provide insight into the uptake mechanism of arsenite in rice and strategies for reducing arsenic accumulation in grain for enhanced food safety.

efflux | influx | arsenic contamination | silicon | aquaporin

Arsenic (As) is a human carcinogen, and there may be no threshold below which it does not cause cancer (1). More than 40 million people worldwide are at risk from drinking As-contaminated groundwater (2), and chronic inorganic As poisoning has reached a massive scale in Bangladesh and West Bengal, India (3). In these countries, As-contaminated groundwater is also widely used for irrigating crops during dry season rice production, adding >1,000 metric tons of As to soil per year in Bangladesh alone and resulting in As accumulation in soils and elevated As uptake by crops (4–6). Elevated As accumulation in rice has the potential to become a new disaster for the population in Southeast Asia (7). As concentrations in rice grain are often high enough to cause concern even in uncontaminated soils containing background levels of As, because paddy rice appears to be particularly efficient in As assimilation compared with other cereal crops (8). Worldwide market surveys show that rice grain contains considerably higher levels of inorganic As than other foods (9, 10). Human intake of As from consumption of rice can be substantial, especially for people who consume a lot of rice (11). It is therefore crucial that the mechanism of As accumulation in rice is understood to counteract this widespread contamination of the food chain.

Plants take up arsenate, the predominant form of As in aerobic soils, through phosphate transporters (12, 13). However, in paddy soils, which are flooded during much of the rice growing season, arsenite becomes the predominant chemical species of As (14). It has been shown that arsenite is taken up via aquaglyceroporins in microbes (13, 15–17). Evidence from physiological studies suggests that arsenite may also be transported by aquaporins in rice (18), although the exact mechanism of arsenite uptake has not been identified in higher plants. Here we report two types of arsenite transporters in rice and their role in As accumulation in rice shoots and grain.

Results and Discussion

NIP Transporters Mediate Arsenite Influx in Rice Roots. Plant aquaporins transport neutral molecules such as water, glycerol, and urea. These transporters are classified into four major subfamilies based on their homology and localization: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic membrane proteins (NIPs), and small and basic intrinsic proteins (SIPs) (19). Arsenite, with a pK_a of 9.2, is present predominantly as uncharged molecules of arsenous acid [$As(OH)_3$] at pH < 8. Recently, a NIP transporter (Lsi1) for silicic acid, which is also uncharged at pH < 8, has been identified in rice (20). Furthermore, arsenous acid [diameter 4.11 Å; $As(OH)_3$ is a tetrahedron with the largest dimension being between the two neighboring OH groups, modeled using WebLab Viewer, www.marcsaric.de/index.php/WebLab_Viewer_Lite] has a molecular size similar to that of silicic acid (diameter 4.38 Å). Therefore, we hypothesized that arsenite transport is mediated by the transporters for silicic acid.

We determined the transport activity for arsenite in *Xenopus* oocytes expressing the rice silicic acid transporter Lsi1. Expression of Lsi1 resulted in a 5-fold increase in the arsenite transport activity (Fig. 1*A*). In the oocytes expressing a mutant *lsi1*, which has a single mutation at the position of the 132nd amino acid (20), transport activity for arsenite was not significantly different from the control (water injection) (Fig. 1*A*). Furthermore, Lsi1 was not permeable to arsenate (Fig. 1*A*). We also assayed the transport activity for arsenite in yeast. Yeast expressing Lsi1 showed 3- to 5-fold-higher transport activity compared with the empty vector control (Fig. 1*B*). These results indicate that arsenite could be transported by the silicic acid transporter in rice.

We then compared As accumulation between the wild-type rice and a mutant defective in silicon uptake, *lsi1* (21). The concentrations of As in the mutant shoots and roots were 71% and 53%, respectively, lower than those of the wild type in the absence of silicic acid after exposure to 2 μM arsenite for 1 day ($P < 0.001$; Fig. 1 *C* and *D*). The addition of silicic acid to the medium significantly ($P < 0.01$) decreased the As concentrations in both roots and shoots of the wild-type plants but not in the mutant. Because the decreased accumulation of As in the mutant shoots may also result from an effect on As translocation from roots to shoots, we compared short-term (30-min) arsenite uptake by the wild-type and mutant roots. Results showed that As uptake by the mutant roots was 57% lower than that by the wild-type roots [$P < 0.01$; supporting information (SI) Fig. S1], indicating a role of Lsi1 in arsenite influx to roots. These results

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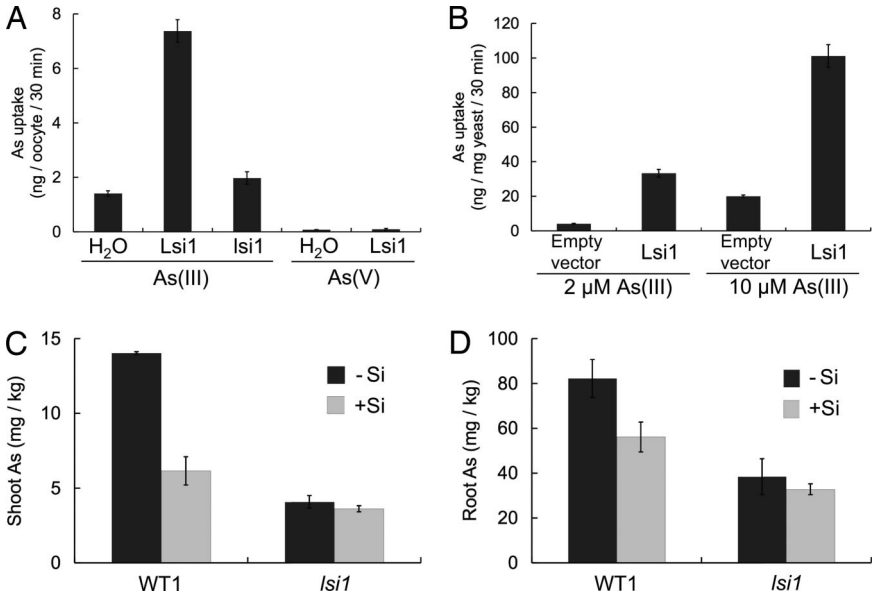

Fig. 1. Arsenite transport activity of Lsi1 in oocytes, yeast, and rice. (*A*) As uptake by oocytes expressing Lsi1 or mutant lsi1, with H_2O as the control. Oocytes were exposed to 100 μM arsenite or arsenate for 30 min. (*B*) As uptake by the yeast mutant (*acr3*) expressing Lsi1 or the empty vector. Yeast was exposed to 2 or 10 μM arsenite for 30 min. (*C* and *D*) Concentration of As in shoots (*C*) and roots (*D*) of the wild-type rice (WT1 = cv. Oochikara) and the *lsi1* mutant. Two-week-old seedlings were exposed to a nutrient solution containing 2 μM arsenite with or without 0.5 mM silicic acid for 1 day. Data are means ± SD (n = 3).

are consistent with the oocyte and yeast results (Fig. 1 *A* and *B*), confirming that Lsi1 is permeable to arsenite *in planta* and is responsible for the primary uptake of arsenite from the external solution to the root cells.

There are 10–13 members of the NIP family in the rice genome (22). Functional analyses of NIPs have revealed diverse transport functions for different substrates including glycerol (23), lactic acid (24), urea and formamide (25), boric acid (26), and silicic acid (20). The substrate selectivity of aquaporins is mainly controlled by the ar/R (aromatic/arginine) selectivity filter (27, 28), which is located in the narrowest region on the extramembrane part of the pore. Based on the ar/R regions, NIPs have been subdivided into three groups, NIP I, II, and III (29). To test whether other NIP members can transport arsenite, we expressed three NIP genes belonging to different groups in oocytes. All members tested showed transport activity for arsenite, although the activity varied among them (Fig. 2*A*). Both Lsi6 (OsNIP2;2) and Lsi1 (OsNIP2;1) belong to NIP III and are permeable to silicic acid, whereas OsNIP1;1 and OsNIP3;1, belonging to NIP I and II, respectively, do not transport silicic acid (29). Arsenous acid is slightly smaller than silicic acid, which may explain why all three NIP groups are permeable to arsenite but only the NIP III members are permeable to silicic acid.

We further investigated the expression levels of these NIP genes in the roots of the wild-type rice. *Lsi1* showed the most abundant expression among the NIP genes examined, whereas the expression of *Lsi6* was much lower than that of *Lsi1* (Fig. 2*B*). The expression of *OsNIP1;1* and *OsNIP3;1* was very weak. The expression of all genes tested was unaffected by the presence of 5 μM arsenite. Furthermore, knocking out *Lsi6* in a T-DNA insertion line of rice had no significant effect on the As concentrations in shoots and roots compared with the wild type (Fig. 2*C*). This result was further confirmed with a Tos-17 insertion line for Lsi6 (Fig. S2). A short-term (30-min) uptake experiment also revealed that Lsi6 did not play any demonstrable role in arsenite uptake by the roots (Fig. S3). Therefore, although Lsi6 showed transport activity for arsenite when expressed in oocytes, it does not contribute significantly to arsenite uptake by rice roots, probably because of low expression. The expression levels of other rice NIPs have been shown to be very low in the roots (30). Taken together, these data indicate that Lsi1 is the major pathway for arsenite uptake in rice.

An Efflux Transporter for Arsenite in Rice. Lsi1 is localized at the distal side of both exodermis and endodermis cells of rice roots

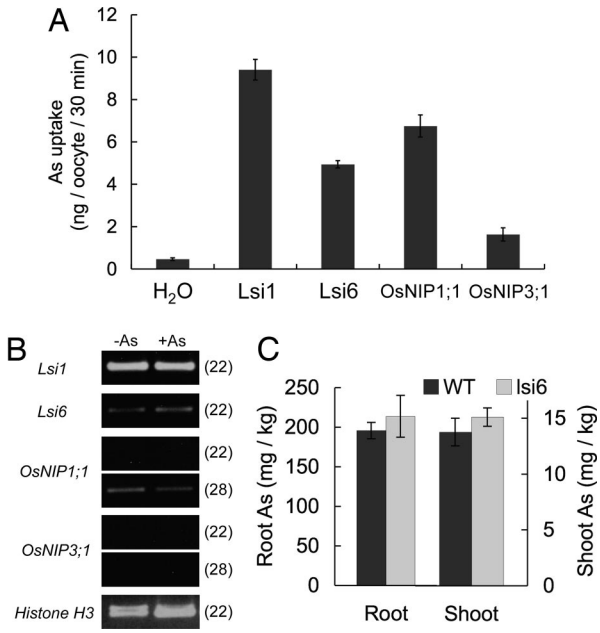

Fig. 2. Arsenite transport by different NIP transporters. (*A*) Arsenite transport activity of NIPs. Oocytes expressing different NIPs were exposed to 100 μM arsenite for 30 min. Data are means ± SD (n = 3). (*B*) Expression level of different NIPs in wild-type rice roots. Seedlings exposed to 0 or 5 μM arsenite for 7 days were used for extraction of RNA. RT-PCR was performed on each sample. Numbers in parentheses are PCR cycles. (*C*) Concentration of As in the shoots and roots in the wild-type rice (cv. Dongjin) and the T-DNA knockout mutant (*lsi6*). Seedlings were exposed to 2 μM arsenite for 7 days. Data are means ± SD (n = 3).

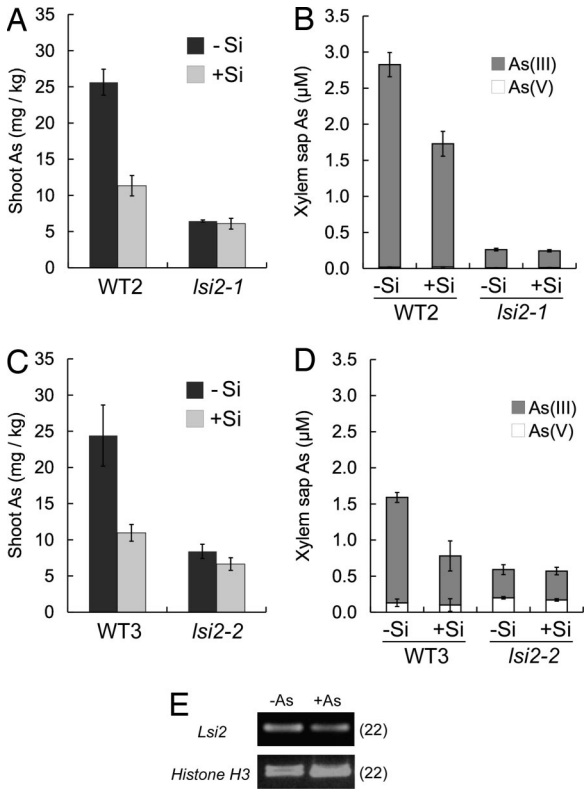

Fig. 3. As transport and expression of *Lsi2*. (*A* and *C*) The concentration of As in the shoots of the two mutants (*lsi2-1* and *lsi2-2*) and their wild-type rice (WT2 = cv. T-65, WT3 = cv. Koshihikari). Two-week-old seedlings of four lines were exposed to 2 μM arsenite with or without 0.5 mM silicic acid for 7 days. (*B* and *D*) The concentrations of arsenite and arsenate in the xylem sap collected from the wild-type rice and *lsi2-1* and *lsi2-2* mutants after seedlings were exposed to 5 μM arsenite with or without 0.5 mM silicic acid for 1 day. (*E*) Expression of *Lsi2* in rice roots. Two-week-old seedlings of the wild-type rice (cv. Nipponbare) were exposed to 0 or 5 μM arsenite for 7 days, and *Lsi2* expression was quantified by RT-PCR. Data in *A*–*D* are means ± SD (n = 3).

and mediates the influx of silicic acid—and arsenite according to the present study—into root cells (20). In rice roots, there are two Casparian bands on the exodermis and endodermis, separated by the aerenchyma formed from the destruction of almost all cortex cells except the exodermis and the endodermis. Therefore, to reach the stele, As must be transported sequentially into and out of the exodermis and the endodermis cells. Arsenite is the predominant form of As in rice roots, regardless of whether arsenate or arsenite is supplied to the plants (31), and is therefore the likely form transported into the stele. Recently, an efflux transporter (Lsi2) for silicic acid has been identified from rice roots (32). In contrast to Lsi1, Lsi2 is localized at the proximal side of both exodermis and endodermis cells of rice roots and mediates the efflux of silicic acid from the exodermis and endodermis cells toward the direction of the stele. To investigate whether Lsi2 can transport arsenite, we compared As accumulation between the wild-type rice and a mutant (*lsi2*) defective in silicic acid transport (32). The As concentration of the mutant shoots was 75% lower than that of the wild-type shoots in the absence of silicic acid ($P < 0.001$; Fig. 3*A*). The presence of silicic acid decreased the As concentration in the wild-type shoots significantly ($P < 0.001$), but not in the mutant. Arsenite was the main As species found in the xylem sap from both the wild type and the *lsi2* mutant, and its concentration in the latter was only 9% of the former ($P < 0.001$; Fig. 3*B*). In contrast to the *lsi1* mutant, there was no significant difference in the short-term (30-min) uptake of arsenite between *lsi2* and the wild-type rice (Fig. S4), suggesting that Lsi2 is involved not in the influx of arsenite into roots, but rather in the efflux of arsenite toward the xylem.

To examine the efflux transport activity of Lsi2 for arsenite, we expressed Lsi2 in the oocytes, yeast, and *Escherichia coli*. However, we failed to detect transport activity in these assay systems. Our previous study showed that Lsi2 did not function for silicic acid transport in the yeast assay system but did function in the oocytes. The failure to observe arsenite transport activity in oocytes may result from the toxicity of arsenite that was injected into the cells or the binding of arsenite to thiols and sulfhydryl groups in proteins.

To confirm that Lsi2 is an efflux transporter for arsenite, we took an alternative approach by isolating an allele of *lsi2* rice mutant. From the M_3 seeds of the rice cultivar Koshihikari irradiated with gamma rays, we obtained a new mutant (*lsi2-2*) having low silicon uptake (Fig. S5). Comparison of the sequence of *Lsi2* between the wild-type rice and the mutant revealed that one nucleotide substitution occurred in the mutant, resulting in one amino acid change at 342 from T to R (Fig. S6). When this mutant was exposed to arsenite, the As concentration in the shoots was 66% lower than that of the wild-type in the absence of silicic acid ($P < 0.001$; Fig. 3*C*), and the arsenite concentration in the xylem sap was 73% lower than that of the wild type ($P < 0.001$; Fig. 3*D*). Addition of silicic acid to the medium significantly ($P < 0.001$) decreased both shoot As concentration and xylem sap arsenite concentration in the wild type but not in the mutant (Fig. 3 *C* and *D*). The results from the two independent *lsi2* mutants indicate that Lsi2 is involved in arsenite transport out of the root cells toward the stele. The expression of *Lsi2* in the wild-type roots was abundant and not affected by arsenite exposure (Fig. 3*E*).

Lsi2 Plays a Crucial Role in Controlling As Concentration in the Rice Grain. To investigate the role of the Lsi1 and Lsi2 transporters in the accumulation of As in rice gain, we grew the three rice mutants with their corresponding wild-type cultivars in a field experiment on a soil containing a background level of total As (5.3 mg/kg). All three mutants had lower concentrations of As in straw than their wild type, with the difference being particularly pronounced in the two *lsi2* mutants, which were only 13–19% of the corresponding wild-type rice ($P = 0.06$ for the WT1/*lsi1* comparison, $P < 0.01$ for the WT2/*lsi2-1* and WT3/*lsi2-2* comparisons; Fig. 4*A*). In the grain and husk, there was no significant difference in the As concentration between the *lsi1* and its wild-type rice (Fig. 4 *B* and *C*). In contrast, in both *lsi2* mutants, the grain As concentration was significantly ($P < 0.01$) lower than the wild-type rice (Fig. 4*B*), being 63% and 51%, respectively, of the corresponding wild-type rice. These results indicate that Lsi2 plays a more important role than Lsi1 in As transport to the shoots and ultimately to the grain in rice and strongly suggest that the root-to-shoot translocation is the key step in controlling As accumulation in shoots.

As transport mechanisms have been studied extensively in bacteria and yeast (33, 34). In microbes, arsenite enters the cell through aquaglyceroporin channels. In the present study, we found that arsenite is transported by aquaglyceroporin NIPs in rice. In bacteria, arsenite is pumped out of the cells by ArsB or ArsAB functioning as an $As(OH)_3$-H^+ antiporter or ATP-driven extrusion pump, respectively (33). In yeast, arsenite is transported out of the cells by Acr3p, a plasma membrane-located arsenite efflux transporter (33). Rice Lsi2 has a similarity to *E. coli* ArsB (18% identity). Although the arsenite transport system in rice appears to be similar to that in bacteria at the cell level, there is a fundamental difference between the two at the organism level: bacteria efflux arsenite for detoxification,

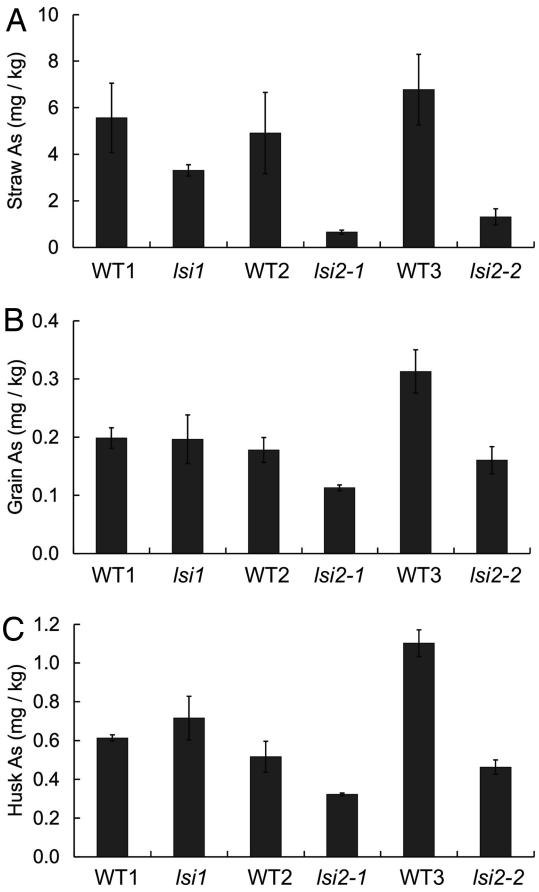

Fig. 4. The concentrations of As in straw (*A*), grain (*B*), and husk (*C*) of field-grown rice. The three mutants and their wild-type rice were grown in a field in 2007. Data are means ± SD ($n = 3$).

whereas arsenite efflux mediated by Lsi2 in rice leads to accumulation in shoots and grain.

In conclusion, for the first time, to our knowledge, in higher plants, we have identified both influx and efflux transporters of arsenite in rice roots, which are involved in arsenite uptake and accumulation. High expression of Lsi1 and Lsi2 in rice leads to a large accumulation of silicon, which benefits yield production (20, 32) but unfortunately also enhances As accumulation in rice shoots and grain. Our results provide important information for developing strategies to reduce As concentration in rice for enhanced food safety. Future research is needed to identify allelic variations in Lsi1 and, particularly, Lsi2 that can favor uptake of silicon over arsenite. At a more practical level, ensuring sufficient silicon availability in the soil is likely to suppress As accumulation in rice.

Materials and Methods

Plant Materials and Growth Conditions. Three mutants (*lsi1*, *lsi2-1*, and *lsi2-2*) and their corresponding wild-type rice (cv. Oochikara, T-65, and Koshihikari), T_2 homozygous seeds of T-DNA insertion line (4A-01373), and T_2 homozygous seeds of Tos17 insertion line (NG0012) for Lsi6 were used in the present study. Mutants *lsi1* and *lsi2-1* were isolated previously (20, 21, 32), and *lsi2-2* was isolated as described below. Seedlings were cultured hydroponically as described previously (21). The solution was renewed every 2 days. All experiments were conducted with three replicates in a glasshouse at 25°C with natural light.

As Treatment. Two-week-old seedlings were exposed to a nutrient solution containing 2 μM arsenite with or without 0.5 mM silicic acid. The treatment solutions were changed once every 2 days, during which period arsenite was stable in the nutrient solution (31). After 1 or 7 days, the roots were washed three times with an ice-cold solution containing 1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2$, and 5 mM MES (pH 5.6) to remove apoplastic As and separated from shoots. The samples were dried at 70°C in a oven for 2 days, digested with HNO_3, and analyzed for As by using inductively coupled plasma MS (ICP-MS) as described in ref. 31. Analysis of variance was performed to test the significance in the difference between wild types and mutants and between Si treatments.

For the collection of xylem sap, two-week-old rice seedlings were exposed to a nutrient solution containing 5 μM arsenite with or without 0.5 mM silicic acid. After 24 h, stems were cut at 1 cm above the roots. The cut surfaces were rinsed with deionized water and blotted dry. Xylem exudates were collected by micropipette for 1 h. As species in the xylem sap were analyzed immediately by using HPLC-ICP-MS as described in ref. 31.

Transport Activity Assay. We used both oocyte and yeast expression systems for the determination of arsenite transport activity. Oocytes were isolated from adult female *Xenopus laevis* frogs and placed in a modified Birth's saline (MBS) solution (88 mM NaCl/1 mM KCl/2.4 mM $NaHCO_3$/15 mM Tris·HCl, pH 7.6/0.3 mM $Ca(NO_3)_2$/0.41 mM $CaCl_2$/0.82 mM $MgSO_4$/10 $\mu g \cdot ml^{-1}$ sodium penicillin/10 $\mu g \cdot ml^{-1}$ streptomycin sulfate). Oocytes were then treated with 0.1% collagenase type B (Roche Diagnostics) in a Ca-free MBS for 1.5 h to remove follicular cell layers, washed five times with MBS free of collagenase, and selected according to the size and development stage. Selected oocytes were incubated for 1 day in MBS at 18°C until the injection of cRNA. The ORFs of different genes including *Lsi1*, mutant *lsi1*, *Lsi6*, *OsNIP1;1*, and *OsNIP3;1*, were amplified and cloned as described before (29), and the cRNA with cap analog was synthesized with mMASSAGGE mMACHINE High Yield Capped RNA Transcription Kit (Ambion) according to the manufacturer's instructions. Fifty nanoliters of cRNA (1 $ng \cdot nl^{-1}$) were injected into the selected oocytes by using a Nanoject II automatic injector (Drummond Scientific). As a negative control, 50 nl of RNase-free water was injected. After incubation in MBS at 18°C for 1 day, the oocytes were exposed to the MBS buffer (pH 7.8) containing 0.1 mM $NaAsO_2$ or 0.1 mM $Na_2HAsO_4{}'7H_2O$. After 30 min of exposure, the oocytes were washed five times with MBS without As and homogenized with 500 μl of 0.1 M HNO_3. As concentrations were determined by ICP-MS. Experiments were performed with three replicates.

For the transport assay using the yeast expression system, the *Lsi1* cDNA was cloned into yeast expression vector pYES2. The arsenite sensitive mutant strain (WΔ*acr3*) was grown at 30°C in a complete YPD medium supplemented with 200 μM G418. Transformation reactions were performed by using S.c.EasyComp Transformation Kit (Invitrogen). One colony was selected in each transformation strain and grown in the liquid YPD medium. The precultured yeast was adjusted to an OD_{600} value of 2.0. Two or 10 μM arsenite was then added to the medium. After 30 min of incubation with gentle shaking, the culture solutions were washed three times with the YPD solution without arsenite and digested with HNO_3. As concentrations were determined by ICP-MS. Each treatment was replicated three times.

Isolation of an Allele of Mutant *lsi2-2*. We used M_3 seeds of rice (*Oryza sativa* L. cv. Koshihikari) irradiated with gamma rays for 10 days for isolation of an allele of mutant *lsi2*. The screening method is based on the tolerance to germanium toxicity (21). We sowed the seeds on a soil containing 50 mg of Ge per kilogram of soil as GeO_2. After 1 month of cultivation, we selected seedlings that did not show the Ge toxicity symptoms of brown spots on the leaf blades. We then grew the candidate mutants in a field and performed a second screening by determining silicon uptake. From the candidate mutants, we further amplified ORF of *Lsi2* by PCR with primers designed based on the sequence of *Lsi2* and sequenced by ABI PRISM 310 Genetic Analyzer using BigDye Terminators V3.1 cycle Sequencing Kit. Finally, we obtained a new mutant (named *lsi2-2*) that is allelic to *lsi2* (named *lsi2-1*).

RNA Isolation and RT-PCR. To examine the expression level of different NIPs in rice roots and the effect of arsenite on the gene expression, we exposed the seedlings of a wild-type rice (cv. Nipponbare) to 5 μM arsenite for 7 days. Then, roots were cut and frozen in liquid nitrogen. Total RNA was extracted by using the RNeasy Mini Kit (Qiagen). One microgram of total RNA was used for first-strand cDNA synthesis using a SuperScript II kit (Invitrogen), following the manufacturer's instructions with an oligo$(dT)_{12–18}$ primer. One-twentieth of the reaction volume was used as template for PCR of *Lsi1*, *Lsi2*, *Lsi6*, *OsNIP1;1*, and *OsNIP3;1*, and *HistoneH3* was used as an internal control. The reactions were run for 22–28 cycles, depending on genes. The primer sequences for PCR of different genes are as follows: *Lsi1*, 5′-CGGTGGATGTGATCGGAACCA-3′ (forward) and 5′-CGTCGAACTTGTTGCTCGCCA-3′ (reverse); *Lsi6*, 5′-GAGTTCGACAACGTCTAATCGC-3′ (forward) and 5′-AGTACACGGTACATGTATACACG-3′ (reverse); *OsNIP1;1*, 5′-CTGATTGCTGGGCCGATCTCG-3′ (forward) and 5′-GCAGTAGTAGTACTGGCAGTAG-3′ (reverse); *OsNIP3;1*, 5′-GTTGCAAGAAGAGGAGAGCAAG-3′ (forward) and

5′-CGAAGAAGATGAGGATGAACGTC-3′ (reverse); *Lsi2*, 5′-ATCTGGGACTTCATGGCCC-3′ (forward) and 5′-ACGTTTGATGCGAGGTTGG-3′ (reverse); *HistoneH3*, 5′-AGTTTGGTCGCTCTCGATTTCG-3′ (forward) and 5′-TCAACAAGTTGACCACGTCAC G-3′ (reverse).

Field Experiment. A field trial was carried out in 2007 at the Experimental Farm of Okayama University. The properties of soil used have been described previously (20). Twenty-day-old seedlings of the three mutants and their wild-type rice prepared as described above were transplanted in the field in mid-June and grown under flooded conditions. Each plot (0.7 m × 0.7 m) contained 36 seedlings, and three replicates were included for each line. At the beginning of October, straw and grain were harvested and separated. The grains were dehusked with a small thresher (Kett TR-110). The straw, grain, and husk samples were digested with HNO_3 and analyzed for As concentration by ICP-MS.

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